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TITLE: Enduring Effects of Traumatic Stress on Brain Neuropeptide Y (NPY) and Corticotropin-Releasing Factor (CRF) Systems: Molecular and Neuropharmacologic Studies

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The funding period allowed us to: 1) further validate repeated social defeat as a model of post-traumatic stress, and 2) demonstrate that the social defeat model is associated with central elevations of CRF levels, as evidenced in CSF samples, as well as a blunted active-phase corticosterone-rhythm, neuroendocrine changes that have been described in some patients with PTSD. Moreover, both neurochemical and pharmacological data strongly implicated a role for amygdala Ucn 3-CRF2 systems in the response to social defeat, with intra-amygdala administration of a CRF2 agonist increasing dominance behavior in animals that had experienced social conflict. Data did not indicate strong adaptations in NPY/Y1/Y2 systems of the amygdala or nucleus accumbens in the defeat model. In contrast, novel data also implicated increases in dynorphin-kappa opioid receptor signaling in the nucleus accumbens in responses to social defeat. Further study of the functional significance of the amygdala-CRF2 and nucleus accumbens-kappa opioid receptor system for post-traumatic stress-symptoms is warranted.

15. SUBJECT TERMS
Post-traumatic stress disorder, animal model, rat, neuropeptide, corticotropin-releasing factor, neuropeptide Y, anxiety, depression, behavior, treatment, gene expression.

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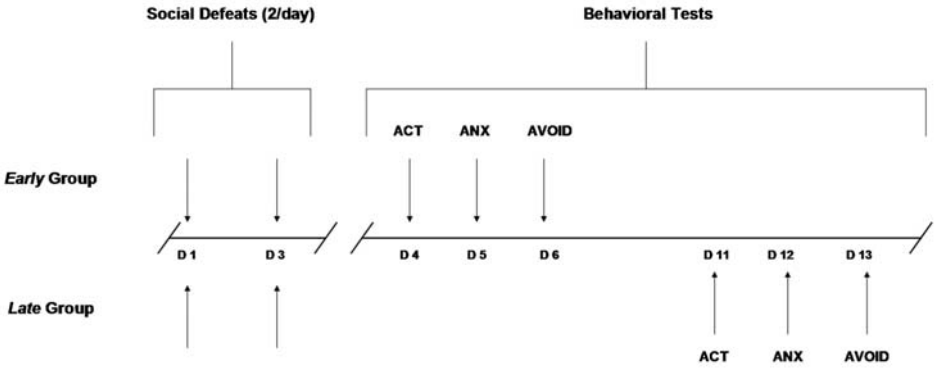
INTRODUCTION:

The present studies sought to further characterize a model of lasting traumatic stress using neuropharmacological and molecular techniques in order to better understand the therapeutic relevance of stress-related peptide systems to the long-term behavioral outcomes of traumatic stress. In particular, the research sought to bridge a gap between: 1) preclinical evidence that neuropeptide Y (NPY) and corticotropin-releasing factor (CRF) systems acutely modulate stress and dysphoria responses and 2) suggestive, but functionally ambiguous, clinical findings that patients with post-traumatic stress disorder (PTSD) show altered NPY and CRF levels. The studies involved examining the effects of traumatic stre ss using a novel rodent model of repeated social defeat, with construct and face validity for many endpoints observed long after post-traumatic stress and in PTSD itself. The objectives/specific aims were: 1) To identify neuroadaptations in extrahypothalamic stress systems, especially the stress-related peptidergic systems such as CRF, NPY and kappa opioid systems following social defeat and their functional relation to behavioral outcome of defeated subjects, and 2) to determine whether manipulation of CRF or NPY systems could modulate the behavioral sequelae of traumatic stress. Further behavioral characterization of the model was performed in the course of the studies.

BODY:

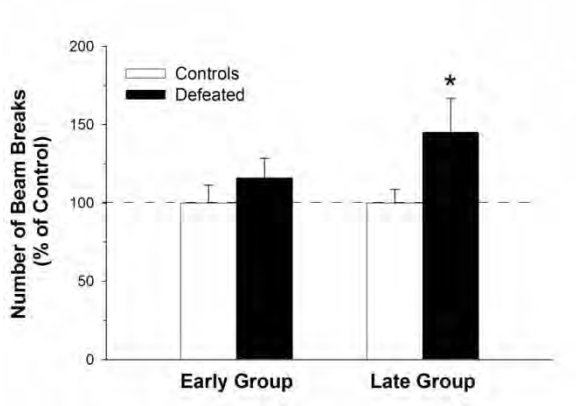
Over the 18-month funding period, substantial progress was made in further behavioral and neuroendocrine characterization of the social defeat model and in identifying molecular changes in the CRF, NPY and kappa opioid receptor systems that result from social defeat within the amygdala and nucleus accumbens, key components of brain stress and brain reward pathways. Identification of mRNA changes were accomplished using real-time PCR analysis with SybrGreen detection and identification of changes at the protein level for ligands were explored using immunohistochemistry and combined *in situ* hybridization/immunohistochemistry. Some of the results already have been published in a peer-reviewed journal (see Appendix for publication), and results also were presented at the 2009 DoD-organized Military Health Research Forum in Kansas City (see Appendix for powerpoint presentation).

I. CHARACTERIZATION OF THE PTSD MODEL. A social defeat model was used, based on the resident-intruder model, wherein an experimental intruder rat is introduced into the cage of a veteran, aggressive, territorial resident. The model has relevance for combat-related post-traumatic symptoms in that the intruders are placed in unfamiliar territory and subjected to perceived threat of physical injury. Rats are studied following repeated defeat or following a single social defeat. A schedule of repeated defeat is schematized below and involves rats receiving 4 social defeats over a 3-day window – with 2 received on Day 1 and 2 received on Day 3. Following emission of the defeat response, intruders are maintained within a wire-mesh enclosure for an additional 60 minutes to receive conspecific threat of physical injury. "Act" refers to tests of locomotor activity during the light cycle (the rats' "sleep-phase"), "Anx" refers to tests of anxiety-like behavior in the elevated plus-maze, and "Avoid" re fers to tests of avoidance-like behavior of an anise extract odor cue that had been present throughout the defeat experiences. Tests were performed in battery format within a given group (Early vs. Late), with different animals used for each group to avoid repeated testing:

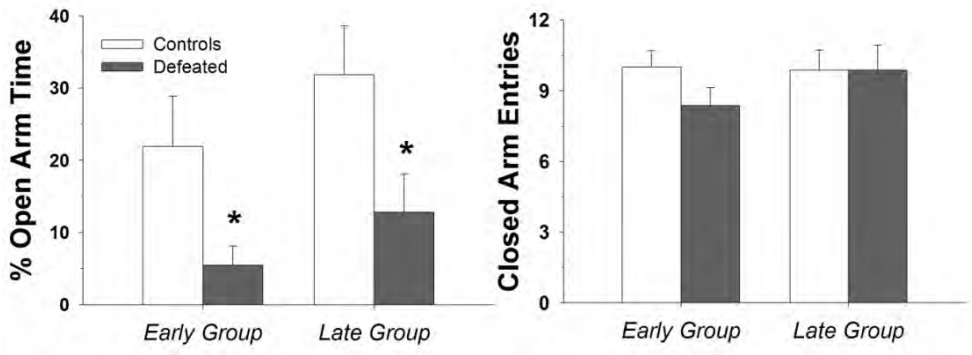


The below pictures illustrate the typical dynamics of the defeat interaction (left) and the continued threat experience (right). The “resident” is the hooded Long-Evans rat, a strain selected for greater territorial aggression, and the “intruder” is the male albino Wistar rat.

In terms of diurnal (sleep-phase) locomotor activity, subjects in this model showed increased locomotor activity, as operationalized by photocell interruptions in wire-mesh locomotor activity cages in which they were residing. This effect was more pronounced 8 days following the final defeat than 1 day following the final defeat as illustrated below. Results are summarized as mean (+SEM) of control levels of beam breaks. As shown in Fig. 5, rats with a history of defeat showed increased sleep-phase locomotor activity The two-way ANOVA performed on the individual beam breaks revealed a significant effect of the stress history during the 11-hr of the light cycle [Stress: $F(1,27) = 4.19$, $p < 0.05$]. This effect tended to be more evident on the Late testing day (11 days post-defeat) When the one-way ANOVA was run for the individual time points, it revealed a significant effect of Stress in the late time point [$F(1,14) = 3.79$, $p < 0.05$], but not in the early time point [$F(1,13) = 0.86$, n.s.], although the Stress*Day interaction did not reach significance:



As shown on the subsequent page, subjects with a history of defeat also showed increased anxiety-like behavior in the elevated plus-maze test, as shown by a decreased percent of arm time spent in the open arms [Stress: $F(1,28) = 9.91$, $p < 0.01$] and a decreased number of entries into the open arms [Stress: $F(1,28) = 6.25$, $p < 0.05$] (not shown). The magnitude of increased anxiety-like behavior was comparable at Early vs. Late post-defeat intervals [Stress*Day: $Fs(1,28) < 0.03$, n.s]. Increases in anxiety-like behavior were not accompanied by non-specific decreases in locomotion [Stress: $F(1,28) = 0.65$, n.s.].



As shown below, rats with a history of repeated defeat also spent less time in a chamber containing the defeat-conditioned odor stimulus as compared to controls [Stress: $F(1,28) = 4.15$, $p < 0.05$] (not shown). The magnitude of this effect was comparable at the Early vs. Late testing time points. In contrast, defeat history did not influence the time spent in the unscented chamber of the Y-maze between groups. Please note that "choice" testing in the chamber involved the 2 of the 3 chambers in the apparatus that subjects exhibited the most similar baseline preference for during a 20-min pretest. This matching/balancing procedure is done in order to reduce confounding influences of baseline aversions or preferences for particular components of the apparatus.

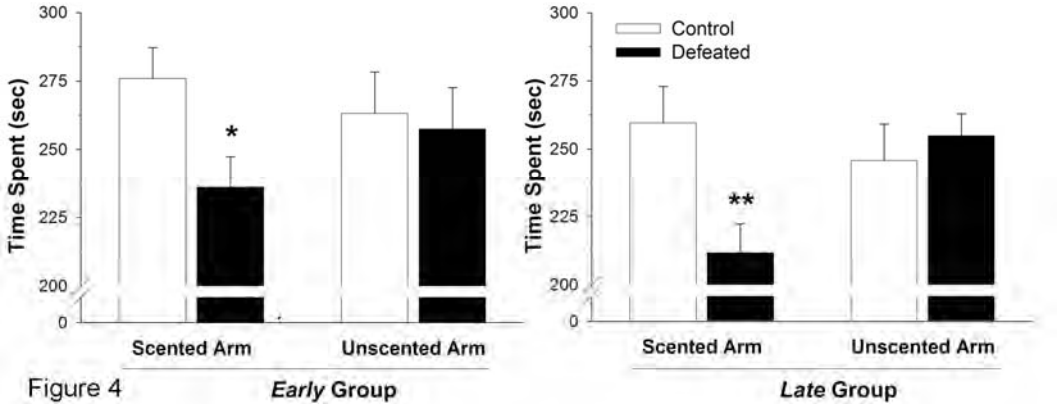
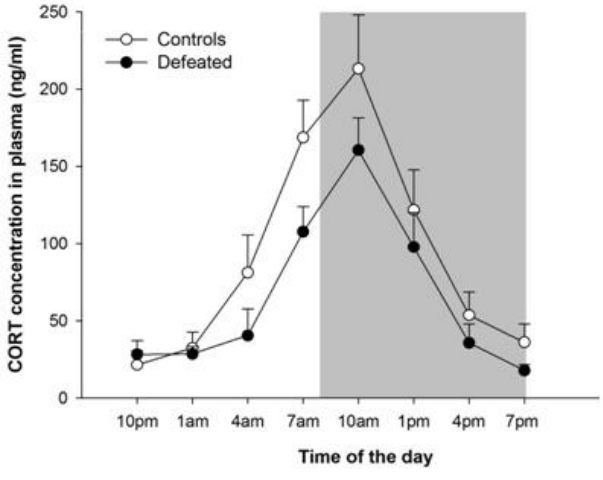
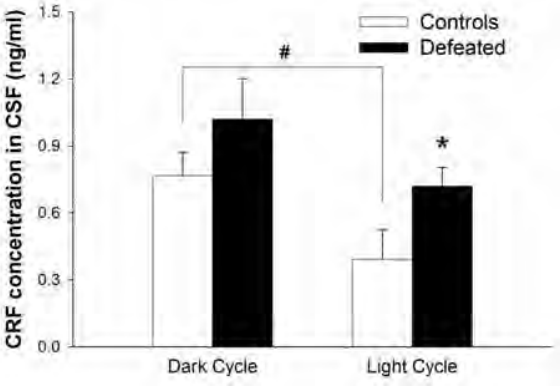


Figure 4

As shown below, these behavioral consequences were accompanied by a blunted plasma corticosterone profile across the circadian cycle (hypocortisolism) at 14 days post-defeat. Tail blood was rapidly sampled at 3-hr intervals and subjected to radioimmunoassay Corticosterone in the cerebrospinal fluid (CSF), rapidly sampled from the cistern magna following induction of anesthesia, was also significantly reduced in both phases of the light-dark cycle (not shown). The results resemble the hypocorticism that has been observed in some studies of patients with PTSD.



As shown below, in contrast to the lower CSF levels of corticosterone, levels of CRF in CSF, as determined by sensitive, specific radioimmunoassay, were significantly elevated in rats 14 days post-defeat during both the light- and dark-phases of the light-dark cycle. The results resemble the elevated CSF CRF that has been observed in some studies of combat veterans with PTSD, and supports the hypothesis of a central activation of CRF circuitry. Insofar as most CSF CRF is thought to be of extrahypothalamic origin, the results specifically suggest an activation of extrahypothalamic CRF circuitry.

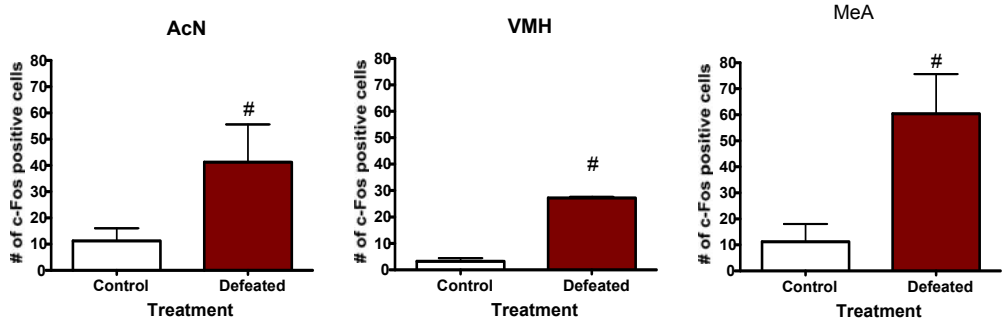


In related studies, we sought to determine whether repeated defeat led to anhedonic-like behavior. Rats (*n*=22/group) were subjected to 3 epochs of defeat experience. Each epoch consisted of a single daily defeat episode as described above on 3 consecutive days. Between each epoch, there was a time-out period of 3 days where no defeat occurred. Therefore, a total of 9 defeats were administered over 15 days (3 Defeat, 3 Timeout, 3 Defeat, 3 Timeout, 3 Defeat). Non-Defeat Controls again were exposed to the same environment with no aggressive resident rat present. Prior to onset of defeat experiences, rats were trained to respond for a saccharin solution in under a progressive ratio reinforcement schedule, and were divided into Defeat and Control conditions matched for baseline performance to ensure comparable breakpoints (maximum number of responses e licited for one reinforcement; Controls: 18.4 + 2.3; Defeated: 18.7 + 1.6). Breakpoints were again measured on the final day of each timeout window between defeat epochs, and a final time 2.5 weeks after the final defeat. After 3 or 6 defeats, there was no difference between Defeated and Control rats in saccharin breakpoint. After 9 defeats there was a significant 30% reduction in breakpoint in the Defeated group relative to Controls, and this was partially attenuated but still significant at 2.5 weeks after the final defeat (data not shown). Treatment with twice daily imipramine (i.p., 2.5 mg/kg) for 2.5 weeks, eliminated the effects of defeat to reduce thresholds. The data indicate that antidepressant-responsive anhedonia-like behavior emerges gradually with increasing defeat experience and persists for at least 18 days once present.

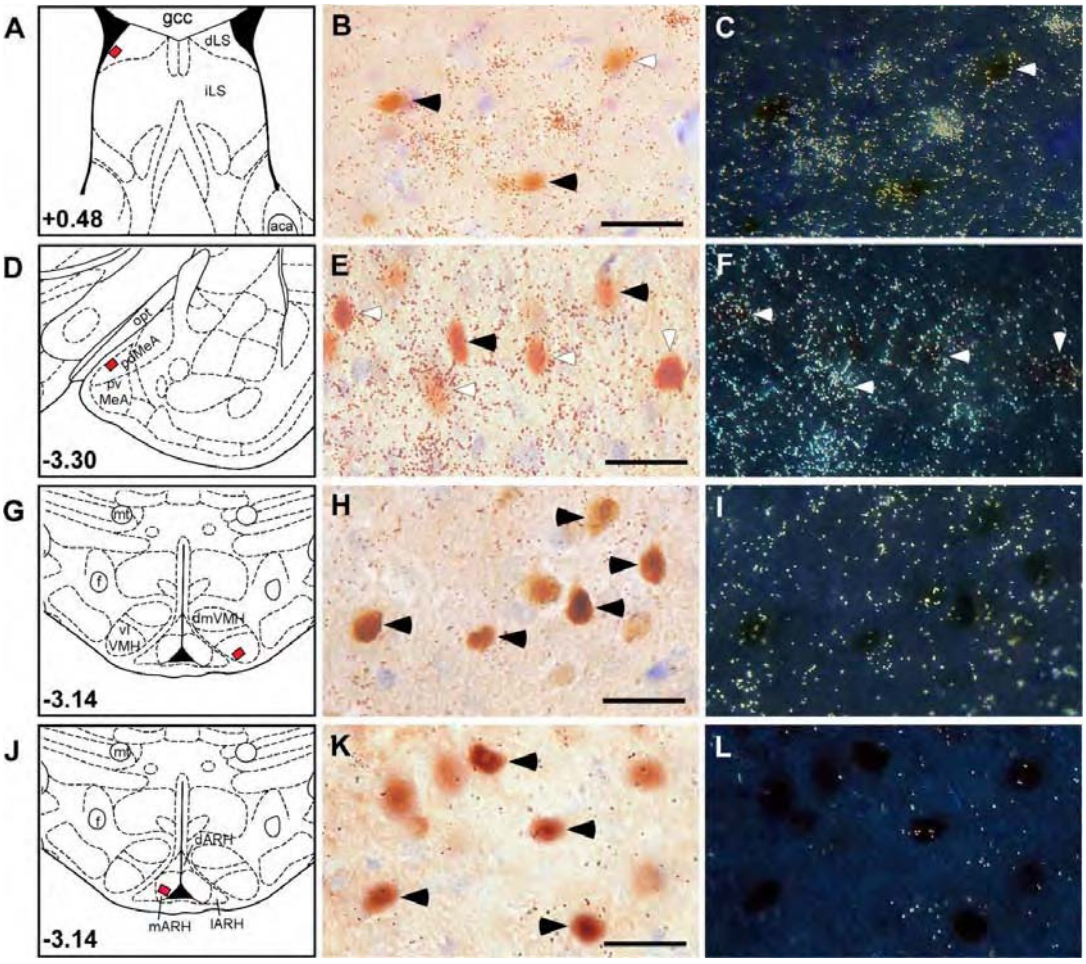
In summary, we have found that repeated social defeat in rats, as administered in the present model, elicits persistent behavioral (anxiety, anhedonia, avoidance, activation during sleep) and neuroendocrine (CRF, CORT) signs that resemble effects of post-traumatic stress and which can be observed at least 2-3 weeks post-stressor.

II. FURTHER ANALYSIS OF STRESS-RELATED NEUROPEPTIDE SYSTEMS IN THE PTSD MODEL. We examined activation of and changes in stress-related CRF, NPY and KOR peptide systems that resulted from social defeat. Type 2 corticotropin-releasing factor receptors (CRF_2) are abundant in brain regions implicated in defeat responses and are putative stress-related molecules. Therefore, one study sought to determine whether neuroactivation and CRF expression co-occurred at brain region or cellular levels following acute defeat. Male “intruder” Wistar rats were placed into the cage of an aggressive “resident” Long-Evans rat (n=6). Upon defeat, intruders (*n*=6) were placed in a wire-mesh chamber and were returned to the resident's

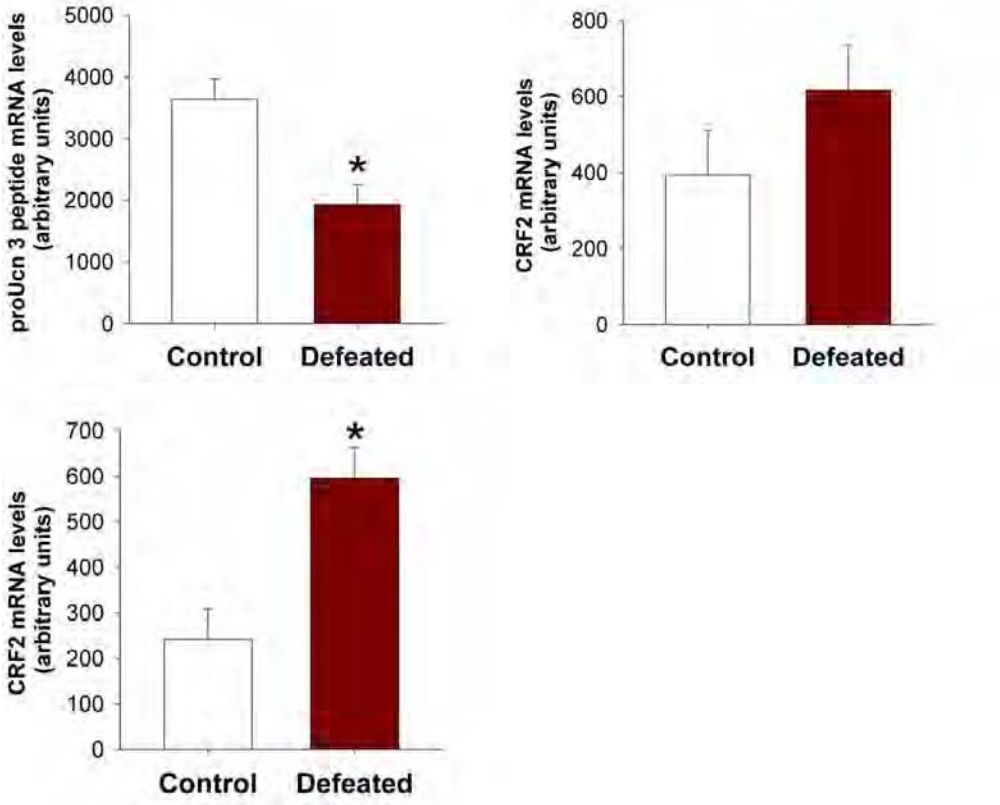
cage for an additional 75 min. Controls (*n*=6) were handled and returned to their home cage for the same duration. Coronal brain sections were stained for an immediate early gene product, Fos, as a neuronal activation marker. As shown below, defeated rats had fivefold, sevenfold, and 10-fold more Fos-positive cells than controls in the arc uate, ventromedial nucleus of the hypothalamus, and medial amygdala post-defeat.



Combined immunohistochemistry with *in situ* hybridization was performed on a subset of brain sections from defeated intruders to visualize Fos immunoreactivity and CRF $_2$ mRNA jointly. As illustrated below, significant colocalization of CRF $_2$ mRNA and Fos-positive cells was observed in the posterior medial amygdala (Panels D, E, and F) but not in the arcuate nucleus (J, K, L), ventromedial hypothalamus (G, H, I), or, to the same degree, lateral septum (A, B, C). The results indicate that CRF $_2$ receptor-positive neurons in the posteri or medial amygdala are involved in the neural response to social defeat. These results were published in the journal *Neuroscience*.

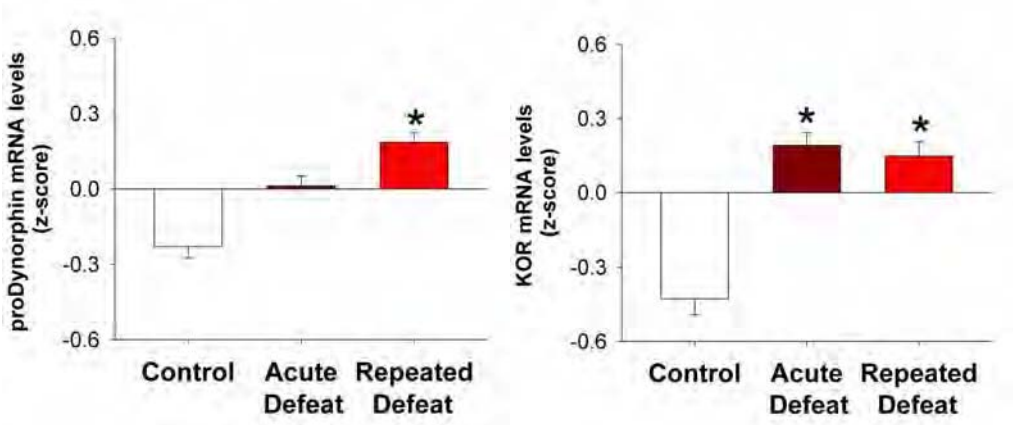


Quantitative real-time PCR was performed to identify changes in gene expression of CRF, NPY or KOR system molecules within discrete limbic br ain regions. As shown below, two hours following acute defeat, subjects exhibited reduced mRNA levels of Ucn 3, a CRF $_2$ agonist, within the medial amygdala (n = 8/group). Increased CRF $_2$ receptor mRNA expression was observed in the central nucleus of the amygdala. A similar, non-significant trend for increased CRF$_2$ mRNA was evident in the medial amygdala (* $p < 0.05$ vs. controls), but no change in CRF $_2$ mRNA expression was observed in the basolateral amygdala or lateral septum, indicating specificity of the CeA effect.



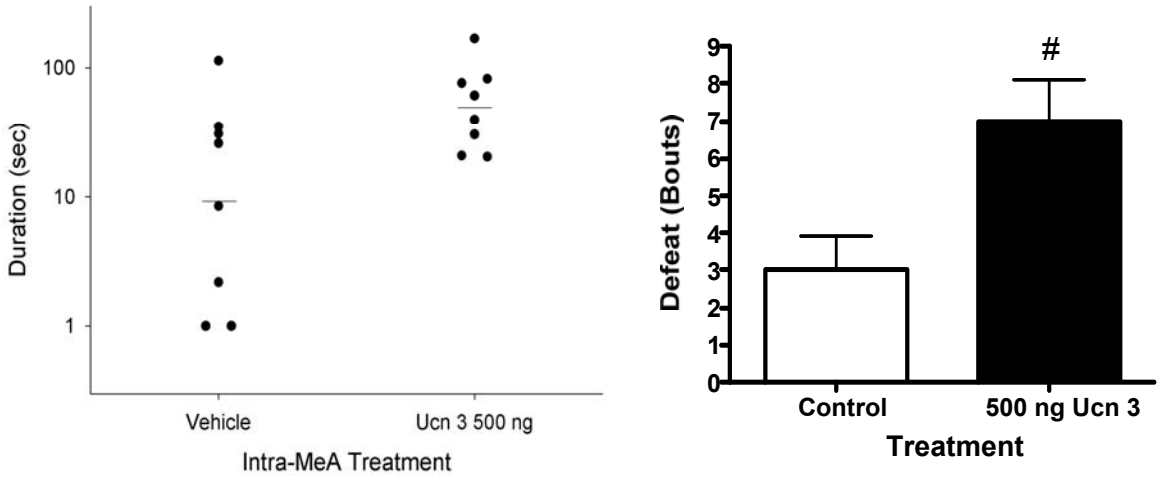
In contrast to the findings with the CRF $_2$ systems, we did not observe significant changes in CRF $_1$ mRNA expression within the medial amygdala, c entral amygdala, basolateral amygdala or lateral septum 2 hr following acute defeat. We also did not observe increases in mRNA expression of the precursor for CRF peptide within the central nucleus of the amygdala, a main site of amygdala CRF synthesis (not shown). Changes in NPY, NPY Y1 and NPY Y2 mRNA expression were not evident in any amygdala nucleus that was studi ed nor in the nucleus accumbens. The results further implicate changes in amygdala CRF $_2$ signaling within the medial and central nuclei of the amygdala in responses to defeat.

To explore another stress-related neuropeptide system that might be involved in responses to defeat stress, we also examined effects of acute and repeated defeat on mRNA expression of the precursor of dynorphin (kappa opioid receptor agonist) and of the kappa opioid receptor within the nucleus accumbens. Kappa opioid receptor signaling in the nucleus accumbens has been suggested to play a role in aversive- and depressive-like responses. As shown below, we observed that the repeated defeat schedule led to significantly elevated gene expression of both proDynorphin and the kappa opioid receptor (KOR) within the nucleus accumbens, with the latter effect also observed 24 hr following a single defeat.

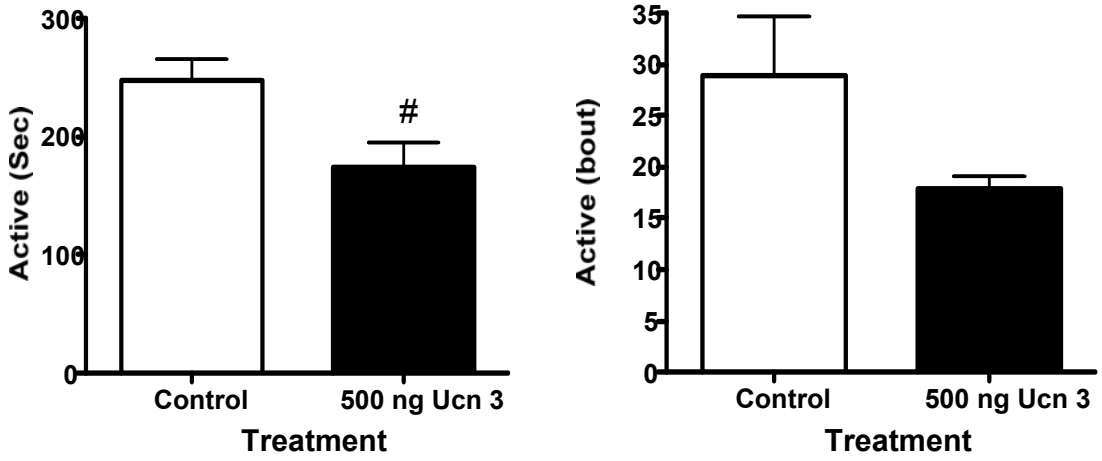


III. PHARMACOLOGICAL MANIPULATION OF NEUROPEPTIDE SYSTEMS IMPLICATED IN SOCIAL DEFEAT. Based on the preponderance of evidence we observed implicating changes in amygdala CRF_2 signaling in the response to social defeat and based on the absence of evidence implicating NPY systems, we sought to determine the effects of intracerebroventricular administration of Ucn 3, a CRF_2 agonist, on dominance/submission responses. Typically, a history of social defeat experiences results in increased submissive and decreased dominance behavior when presented with an unfamiliar conspecific. Based on our observation that social defeat led to downregulation of Ucn 3 mRNA within the medial amygdala, we tested the hypothesis that administration of Ucn 3 into the medial amygdala would increase dominance behavior. Experimental rats were tested in a modified resi dent-intruder paradigm in which they served as residents. Animals were single housed in their home cage without change of bedding to facilitate territorial behavior. After 1 week, rats (*n* = 16) were trained to acquire defeat behavior for 3 days during which an unfamiliar male intruder rat was introduced into the home cage of the experimental resident rat (two 5-min confrontations on each day). On a subsequent 4 th day, experimental resident rats received local administration of Ucn 3 (500 ng) or vehicle into the medial amygdala 10 min before introduction of an unfamiliar, untreated male Wistar rat. Behavior was videotaped and scored by a treatment-naïve rated during the subsequent 10-min confrontation.

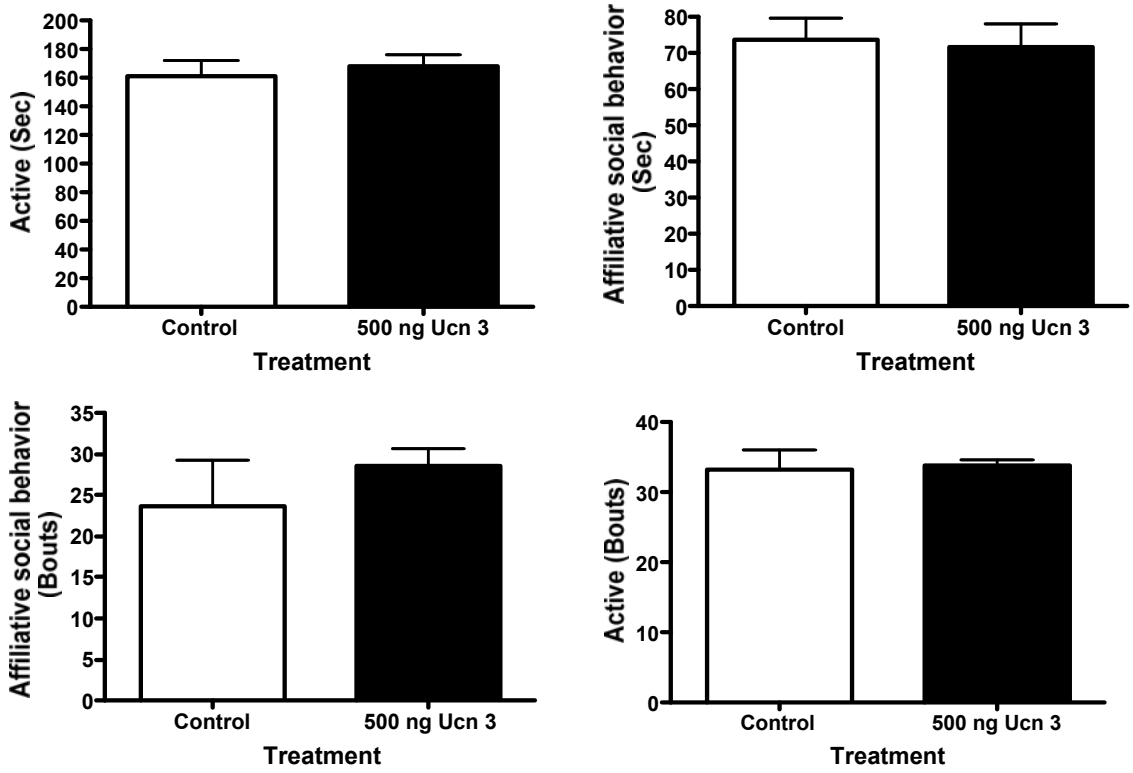
As shown below, rats treated with Ucn 3 in t he medial amygdala exhibited a significantly ($p < 0.05$) greater duration of defeat behavior as well as more than twice as many bouts of defeat than did vehicle-treated subjects.



As shown below, the increase in defeat behavior did not reflect a non-specific behavioral activation because Ucn 3-treated did not exhibit an increase of active locomotion, as defined by the total duration of active movement or the bouts of locomotion. Rather, the duration of active locomotion was even significantly reduced in Ucn 3-treated subjects.



Moreover, as shown below, intra-medial amygdala administration of Ucn 3 to rats without a history of experimental conflict did not alter the affiliative social behavior of rats in a social interaction test performed in a familiar open field. Thus, the results are not simply due to differences in conspecific recognition or non-specific differences in social behavior.



Overall, the results support the hypothesis that medial amygdala Ucn 3-CRF_2 systems modulate dominance/submissive behavior with other conspecifics, with Ucn 3 increasing dominance/defeat behavior. Insofar as increases in submissiveness along the dominance-submission dimension have frequently been suggested to be a component of trauma reactions, especially depressive

responses (Malatynska and Knapp, 2005; Malatynska et al., 2005, 2007; Pinhasov et al., 2007), the current results suggest that further study of the therapeutic potential of modulating amygdala Ucn 3-CRF_2 systems is warranted.

KEY RESEARCH ACCOMPLISHMENTS:

- Behavioral and neuroendocrine studies showed that repeated social defeat in rats, as administered in the present model, elicits per sistent behavioral (anxiety, anhedonia, avoidance, activation during sleep) and neuroendocrine (CSF CRF, CSF CORT, plasma CORT) signs that resemble effects of post-traumatic stress and which can be observed at least 2-3 weeks post-stressor, validating repeated social defeat as a model highly relevant to post-traumatic stress symptoms.

- We used Fos immunohistochemistry to map brain regions activated by social defeat

- We obtained neurochemical evidence using combined immunohistochemistry/*in situ* hybridization and quantitative real-time PCR that strongly implicates amygdala CRF_2 systems in the response to social defeat. We showed that CRF_2 receptor-positive neurons in the posterior medial amygdala are involved in the neural response to social defeat, whereas CRF_2 neurons in the lateral septum, arcuate nucleus and ventromedial nucleus of the hypothalamus were not robustly activated by defeat. - Also consistent with a defeat-induced change in amygdala CRF_2 systems, we found that acute defeat reduced mRNA levels of Ucn 3 in the medial amygdala, while increasing CRF_2 receptor mRNA expression in the central and, to lesser degree, medial amygdala.

- Real-time PCR analysis also demonstrated upregulation of dynorphin and kappa opioid receptor gene expression in the nucleus accumbens following repeated social defeat.

- In contrast, we found, somewhat unexpectedly, that defeat did not acutely alter mRNA expression of NPY or its Y_1 and Y_2 receptors, nor of the CRF_1 receptor in the amygdala or nucleus accumbens.

- Further implicating amygdala CRF_2 systems in adaptations to social conflict, behavioral pharmacologic analyses showed that intra-amygdala administration of urocortin 3 (a CRF_2 agonist), specifically increased dominance behavior, and not other active behaviors, in “resident” animals with a history of social conflict.

REPORTABLE OUTCOMES:

- We published our findings concerning the neuroactivational responses to social defeat and the involvement of medial amygdala CRF_2 receptors therein as follows:

Fekete EM, Zhao Y, Li C, Sabino V, Vale WW, Zorrilla EP, Social defeat stress activates medial amygdala cells that express type 2 corticot ropin-releasing factor receptor mRNA. *Neuroscience*. 2009 Aug 4;162(1):5-13. Epub 2009 Apr 7.

- We presented our findings at the Military Health Research Forum meeting as:

Zorrilla, Eric P., Military Health Research Forum 2009, Neuroadaptations in stress-related peptidergic brain systems following social defeat stress, Kansas City, MO, August 14-16, 2009.

- We have a manuscript pending submission that describes the behavioral and neuroendocrine analyses that validated the repeated social defeat procedure as a model of post-traumatic stress symptoms. Manuscript copy can be sent as follow-up once accepted.

- We have a manuscript pending submission that reports the effects of repeated defeat on Ucn 3 and CRF_2 mRNA expression in the medial amygdala as well as the behavioral effects of intra-amygdala administration of Ucn 3 on dominance behavior. Manuscript copy can be sent as follow-up once accepted.

CONCLUSION:

The funding period allowed us to: 1) further validate repeated social defeat as a model of post-traumatic stress systems that exhibits lasting increases in anxiety-like behavior, anhedonic-like behavior, sleep-phase motor activity, avoidance of conditioned stimuli associated with the trauma context, and 2) demonstrate that the social defeat model is associated with central elevations of CRF levels, as evidenced in CSF samples, as well as a blunted active-phase corticosterone-rhythm, neuroendocrine changes that have been described in some patients with PTSD. Moreover, both neurochemical and pharmacological data strongly implicated a role for amygdala Ucn 3–CRF_2 systems in the response to social defeat, with intra-amygdala administration of a CRF_2 agonist increasing dominance behavior in animals that had experienced social conflict. Data did not indicate strong adaptations in NPY/Y1/Y2 systems of the amygdala or nucleus accumbens in the defeat model. In contrast, novel data also implicated increases in dynorphin-kappa opioid receptor signaling in the nucleus accumbens in responses to social defeat. Further study of the functional significance of the amygdala-CRF_2 and nucleus accumbens-kappa opioid receptor system for post-traumatic stress-symptoms appears to be warranted.

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APPENDICES:

Publication:

Fekete EM, Zhao Y, Li C, Sabino V, Vale WW, Zorrilla EP, Social defeat stress activates medial amygdala cells that express type 2 corticot ropin-releasing factor receptor mRNA. *Neuroscience*. 2009 Aug 4;162(1):5-13. Epub 2009 Apr 7.

Presentation:
Zorrilla, Eric P., Military Health Research Forum 2009, Neuroadaptations in stress-related peptidergic brain systems following social defeat stress, Kansas City, MO, August 14-16, 2009.

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SOCIAL DEFEAT STRESS ACTIVATES MEDIAL AMYGDALA CELLS THAT EXPRESS TYPE 2 CORTICOTROPIN-RELEASING FACTOR RECEPTOR mRNA

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Abstract—Defeat is a social stressor involving subordination by a threatening conspecific. Type 2 corticotropin-releasing factor receptors (CRF_2) are abundant in brain regions implicated in defeat responses and are putative stress-related molecules. The present study sought to determine whether neuroactivation and CRF_2 expression co-occurred at brain region or cellular levels following acute defeat. Male "intruder" Wistar rats were placed into the cage of an aggressive "resident" Long-Evans rat (n=6). Upon defeat, intruders (n=6) were placed in a wire-mesh chamber and were returned to the resident's cage for an additional 75 min. Controls (n=6) were handled and returned to their home cage for the same duration. Coronal brain sections were stained for an immediate early gene product, Fos, as a neuronal activation marker. Combined immunohistochemistry with *in situ* hybridization was performed on a subset of brain sections from defeated intruders to visualize Fos immunoreactivity and CRF_2 mRNA jointly. Defeated rats had fivefold, sevenfold, and 10-fold more Fos-positive cells than controls in the arcuate, ventromedial nucleus of the hypothalamus, and medial amygdala post-defeat. Significant colocalization of CRF_2 mRNA and Fos-positive cells was observed in the posterior medial amygdala but not in the arcuate nucleus or ventromedial hypothalamus. The results indicate CRF_2 receptor-positive neurons in the posterior medial amygdala are involved in the neural response to social defeat. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: corticotropin-releasing factor, CRF_2 receptor, ventromedial or paraventricular hypothalamus, medial nucleus of the amygdala, lateral septum, social defeat stress or resident-intruder test.

Defeat is a social stressor involving subordination by a threatening conspecific. In many species, a single defeat results in lasting autonomic, reproductive, and behavioral changes (Tornatzky and Miczek, 1993; Koolhaas et al., 1997; Blanchard et al., 2001; Korte and De Boer, 2003; Sapolsky, 2005). Victors develop increased offensive behaviors, whereas losers develop increased defensive behaviors and less territorial behavior (Blanchard et al., 1995; Tamashiro et al., 2004; Huhman, 2006; Shimozuru et al., 2006). Rodent subordinates show decreased testosterone production and decreased mounting behavior during chronic social conflict, whereas dominants exhibit normal or elevated testosterone levels (Blanchard et al., 1995; Tamashiro et al., 2004; Huhman, 2006). Even brief exposure to territorial, dominant males leads to weight loss, anorexia, and hyperthermia (Marini et al., 2006); reduced reproduction-relevant social behaviors such as territorial aggression, marking, and mating; and stress-like changes in hypothalamic–pituitary–adrenocortical and gonadal axis activity (Shively and Kaplan, 1984; Yoshimura and Kimura, 1991; Huhman, 2006).

Fos, the protein product of the immediate-early gene c-*fos*, has been used as a cellular marker of brain regions activated by defeat (Miczek et al., 2004). In rats, increased Fos protein or c-*fos* mRNA expression has been reported in the ventral lateral septum (LS), central and medial nuclei of the amygdala (CeA, MeA), bed nucleus of the stria terminalis (BNST), lateral hypothalamus, paraventricular nucleus of the hypothalamus (PVN), dorsal raphe, and hippocampus following acute defeat (Martinez et al., 1998; Gardner et al., 2005; Funk et al., 2006; Calfa et al., 2007). Defeated mice likewise showed higher c-*fos* mRNA in the septum, preoptic area, lateral hypothalamus, amygdala, and dorsal raphe (Stork et al., 1997). Hamsters showed high c-*fos* mRNA expression in the CeA, MeA, BNST, LS, and arcuate nucleus of the hypothalamus (ARH) following one defeat and also in the ventromedial hypothalamus (VMH) after repeated defeat (Kollack-Walker et al., 1997, 1999).

Corticotropin-releasing factor (CRF) family peptides are key mediators of behavioral, autonomic, and neuroen-

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Abbreviations: ARH, arcuate nucleus of the hypothalamus; BNST, bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; CRF, corticotropin-releasing factor; CRF_1, type 1 corticotropin-releasing factor receptor; CRF_2, type 2 corticotropin-releasing factor receptor; cRNA, complementary RNA; dLS, dorsal part of the lateral septum; KPBS, potassium phosphate-buffered saline; LS, lateral septum; MeA, medial nucleus of the amygdala; PVN, paraventricular nucleus of the hypothalamus; SSC, sodium chloride/sodium citrate; VMH, ventromedial hypothalamus.

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docrine responses to stress (Vale et al., 1981). CRF is distributed in the PVN, from which it elicits pituitary adrenocorticotropic hormone release following secretion from the median eminence (Sawchenko et al., 1993), and in extrahypothalamic brain regions where it subserves non-neuroendocrine, stress-related functions (Zorrilla and Koob, 2004). For example, acute defeat elevates CRF mRNA in the hippocampus (Marini et al., 2006), ventral BNST, and CeA (Funk et al., 2006).

Two genes encoding G-protein-coupled CRF receptors have been identified (CRF_1 and CRF_2) (Chen et al., 1993; Lovenberg et al., 1995). Localization studies show distinct distributions of these receptor subtypes, suggesting functional diversity (Fekete and Zorrilla, 2007). The CRF_1 receptor, expressed throughout the brain including the cortex and cerebellum, mediates activational and anxiogenic-like components of stress-related behaviors (Zorrilla and Koob, 2004). CRF_2 receptors are expressed in discrete brain areas, including the hypothalamus and limbic system, with CRF_2 receptors in the hypothalamus and hindbrain regulating food intake and gastric motility (Fekete and Zorrilla, 2007). However, whether and which CRF_2 receptors participate in endogenous responses to stress remain less clear, with data suggesting roles in the LS (Henry et al., 2006) and dorsal raphe (Hammack et al., 2003; Staub et al., 2005; Cooper and Huhman, 2007). Interestingly, CRF_2 receptor mRNA is also present at medium-to-high levels in other brain regions where defeat induces c-*fos* gene expression, including the BNST, MeA, PVN, ARH, and VMH (Van Pett et al., 2000), raising the possibility that CRF_2 neurons may mediate responses to social defeat.

Therefore, the present study tested the hypothesis that CRF_2-containing neurons are activated by social defeat. Fos was used as a marker for neuronal activation to determine semiquantitatively whether social defeat activates neurons in areas that express CRF_2. Combined immunohistochemistry and *in situ* hybridization was then performed to visualize Fos immunoreactivity and CRF_2 mRNA simultaneously to identify neurons that express both Fos and CRF_2.

EXPERIMENTAL PROCEDURES

Subjects

Adult male Wistar rats ($n=12$; 275–300 g on arrival; Charles River, Raleigh, NC, USA) were "intruders" in the resident–intruder defeat model of the present studies. Subjects were single-housed in wire-topped, plastic cages (48×27×20 cm) in a 12-h light/dark (08:00 h lights off), humidity- (60%) and temperature-controlled (22 °C) vivarium. Larger, adult male Long-Evans rats ($n=6$, 450–500 g on arrival) were housed in enclosures (48×69×50 cm) with sawdust-covered, stainless steel floors and were territorial "residents" in the defeat model. Each resident was stably housed with an adult female Wistar rat ($n=6$) that had received electrocauterization of the uterine coils under isoflurane anesthesia (1%–3% in oxygen) to prevent pregnancy. Food and water were available *ad libitum* unless stated otherwise. Procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication 85–23, revised 1996) and the "Principles of laboratory animal care" (http://www.nap.edu/readingroom/bookslabrats) and were approved by the Institutional Animal Care and Use Committee of the Scripps Research Institute. All efforts were made to minimize the number of animals used and their suffering.

Resident-intruder social defeat procedure

Each resident ($n=6$) was housed with a sexually mature Wistar female for 1 month to promote territorial behavior. The female mate was removed from the cage before placing the intruder within the enclosure. Long-Evans rats were used as residents following the precedent of Miczek and colleagues (Miczek, 1979; Tornatzky and Miczek, 1993, 1994), who developed and characterized the resident–intruder model of social defeat. Long-Evans rats are used as residents in the model because of the strain's propensity for territoriality and dominance behavior across the lifespan (Blanchard et al., 1984). Strains that show somewhat less social agonistic behavior are used as intruders in this model; here, intruders were from the less aggressive Wistar strain (Scholtens and Van de Poll, 1987; Schuster et al., 1993), also a well-studied model in neuroscience research. To potentiate dominance behavior, residents were exposed to "training" intruders—post-pubertal, smaller (200–225 g) male Wistar rats—for 16 days before experimental studies. Residents were exposed to different training intruders twice per day, every other day, with exposure lasting until the intruder submitted or, if submission did not occur, 5 min. Intruders were removed from the home cage immediately after defeat, and females were returned to the cage. The criterion for defeat was adoption of a submissive, supine posture by the intruder rat, as defined by Miczek and colleagues (Miczek, 1979; Tornatzky and Miczek, 1993). This training reduces the mean latency by which residents later achieve submissions over experimental intruders to <90 s and thereby reduces the duration of physical conflict that residents require to attain defeats. Potential residents that injured intruders during training, did not achieve 3 consecutive days of defeat, or had mean defeat latencies >120 s were excluded from the study. Training was conducted during the animals' dark cycle under red lighting.

For testing, each experimental intruder ($n=6$) was placed inside the resident's home cage until defeat. Upon submission, intruders were placed inside a protective wire-mesh enclosure (20×20×32 cm) that was then placed within the resident's home cage. Intruders remained in the enclosure for 75 min to allow for Fos protein expression and to provide further psychosocial threat. The wire enclosure prevented injurious physical interactions but allowed auditory, olfactory, visual and limited physical contact (mouth/nose). Control rats ($n=6$) were picked up, briefly handled and returned to their home cage for 75 min.

Perfusion and tissue sectioning

After the 75-min post-defeat interval (within the protective wire-mesh enclosure for intruders or home cage for controls), subjects were anesthetized with an overdose of chloral hydrate (1 g/kg body weight, i.p.) and perfused transcardially with 150 ml of saline followed by 350 ml of 4% paraformaldehyde in borate buffer (pH 9.5). The whole brain was post-fixed in 25% sucrose in 4% paraformaldehyde at 4 °C for 24 h. Brains were stored at −80 °C until use. Coronal sections (25 μm) were cut on a sliding microtome and collected in a one-in-12 series. The tissue sections were stored until use at −20 °C in multiwell tissue culture plates containing cryoprotectant. The first two wells were used for immunocytochemistry. Sections from one defeated rat were not useable for counting Fos-labeled cells in the LS, MeA, ARC or BNST; sections from one control rats were not useable for the ARC or BNST.

Immunocytochemistry

Tissue sections were rinsed in 0.05 M potassium phosphate-buffered saline (KPBS) followed by treatment with 1% $NaBH_4$–

KPBS solution (Sigma, St. Louis, MO, USA). Sections were incubated with Fos protein antibodies raised in rabbit (1:60,000, EMD Biosciences, San Diego, CA, USA) in KPBS with 0.4% Triton X-100 at room temperature for 1 h, followed by 4 °C for 48 h. After incubation, sections were rinsed in KPBS and incubated in biotinylated donkey anti-rabbit IgG (1:600, Vector Laboratories, Burlingame, CA, USA) in KPBS with 0.4% Triton X-100 for 1 h at room temperature, followed by a 1-h incubation at room temperature in avidin–biotin complex solution (4.5 μl of A and B each per ml of KPBS-0.4% Triton X-100; Vectastain ABC Elite Kit, Vector Laboratories). The antibody–peroxidase complex was visualized with a mixture of 3,3-diaminobenzidine (0.2 mg/ml) and 3% H_2O_2 (0.83 μl/ml) in 0.05 M Tris buffer–saline solution. Following the staining, sections were mounted on gelatin-coated slides, dehydrated, and coverslipped. For the Fos protein/CRF_2 mRNA double-labeling study, brain sections were processed for Fos staining as described above, with the exception that all solutions were treated with diethylpyrocarbonate to prevent RNase contamination. Following Fos staining, brain sections were processed for *in situ* hybridization.

In situ hybridization

To determine whether some cells that expressed Fos protein following defeat were CRF_2 receptor-expressing cells, immunocytochemistry and *in situ* hybridization were serially combined to visualize Fos protein and CRF_2 mRNA simultaneously. All solutions were treated with diethylpyrocarbonate to protect brain sections from RNase contamination. For *in situ* hybridization, a [^{33}P]uridine triphosphate-labeled (PerkinElmer, Boston, MA, USA) CRF_2 complementary RNA (cRNA) probe was transcribed from a 460 base-pair 5′-region of the CRF_2 cDNA linearized with XbaI (Li et al., 2002). The riboprobes were directed against the 5′ region of rat CRF_2 receptors, covering the sequence up to the third presumed transmembrane region (Chalmers et al., 1995). The specific activity of the probe was $\sim 5\times10^5$ cpm/ml of hybridization buffer. Following Fos immunostaining, brain sections were washed with KPBS, treated with acetic anhydride and exposed to the CRF_2 cRNA probe overnight in moist chambers at 55 °C. Sections were then washed two times in 4× sodium chloride/sodium citrate (SSC) buffer, in RNase A (30 μg/ml final concentration, Sigma), one time in 2× SSC and finally in 0.1× SSC at 60 °C, and mounted on gelatin-coated slides that were then dipped in NTB-2 emulsion (Eastman Kodak, Rochester, NY, USA). The slides were exposed for 15 days at 4 °C and developed.

Imaging

Slides were examined using a Nikon (E600) light microscope (Lake Forest, CA, USA). The numerical apertures of the 4×, 10× and 40× lens were 0.2, 0.45 and 0.95, respectively. Four brain sections from each region of interest were analyzed, relative to bregma (anterior/posterior), as follows: from +1.2 to 0.6 for dorsal part of the lateral septum (dLS), from +0.48 to 1.3 for BNST, from −1.80 to −3.60 for MeA, from −1.0 to −2.12 for PVN, from −2.1 to −3.30 for VMH and from −1.80 to −3.60 mm for ARH per a rat brain atlas (Paxinos and Watson, 1998). Each coordinate range was further subdivided into 600 μm segments (two segments for PVN and VMH and three segments for dLS, BNST, MeA and ARH). One to two sections were analyzed from each 600 μm segment for data collection. Images were captured by a digital camera (Photometrics Coolsnap CF, Tucson, AZ, USA). Fos-positive cells in captured images were analyzed and counted using Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA). CRF_2 positive cells were identified from the same regions that were analyzed for Fos immunoreactivity as clusters of silver grains, indicating [^{33}P]uridine triphosphate-labeled CRF_2 cRNA probe signals, in dark field photomicrographs. The images were cropped and adjusted to balance brightness and contrast in Adobe Photoshop 8.0 (Adobe Systems, San Jose, CA, USA) before importing the images into Canvas 8.0 (Deneba Systems, Miami, FL, USA) for assembly into plates. The brain plates were then imported into Canvas for reordering.

Statistical analysis

For each rat, Fos-positive cells were counted bilaterally in four brain sections and averaged, with all sections from a given brain region evaluated by a single treatment-naive rater. Data were subjected to Student's *t*-test for comparisons between the control and defeated groups or Welch's *t*-test when variance significantly differed between groups. The software package used was InStat 3.0 (GraphPad, San Diego, CA, USA).

RESULTS

Distribution of Fos-positive cells in selected rat forebrain areas after social defeat

Intruders were rapidly defeated by residents (range: 31–126 s). An initial survey of the forebrain found that defeated rats showed more Fos-positive cells compared with control rats 75 min post-defeat in several regions in the limbic system and the hypothalamus (Table 1). In limbic structures, Fos-positive cells were observed in the dLS, MeA, and anterior cortical nuclei of the amygdala. A few Fos-positive cells were observed in the BNST of defeated subjects, but not more than in controls.

Table 1. Semi-quantitative distribution of Fos-positive cells in selected areas in rat brain 75 min after acute defeat relative to control

Forebrain region	Density of Fos-immunoreactive cells
Septum	
Dorsolateral nucleus	+
Intermediate nucleus medial nucleus	−/+
	−/+
Amygdala	
Anterior cortical nucleus	+
Posterior cortical nucleus	−/+
Basolateral nucleus	−/+
Basomedial nucleus	−/+
Central nucleus	−/+
Medial nucleus	+++
BNST	
Rostral region	−
Posterior region	−
Hypothalamus	
Arcuate nucleus	++/+++
PVN	
Parvocellular	−
Magnocellular	−/+
Supraoptic nucleus	+
VMH	++/+++
Lateral hypothalamus	+

Semi-quantitative ratings reflect the density of Fos-positive cells relative to controls, with (–) representing control-like levels of Fos-labeled cells, and plus symbols indicating slightly (+), moderately (++), or highly elevated (+++) numbers of Fos-labeled cells compared with controls in a given cell group or field. The (–/+) symbol indicates control-like or slightly elevated numbers of Fos-labeled cells, varying across intruders.

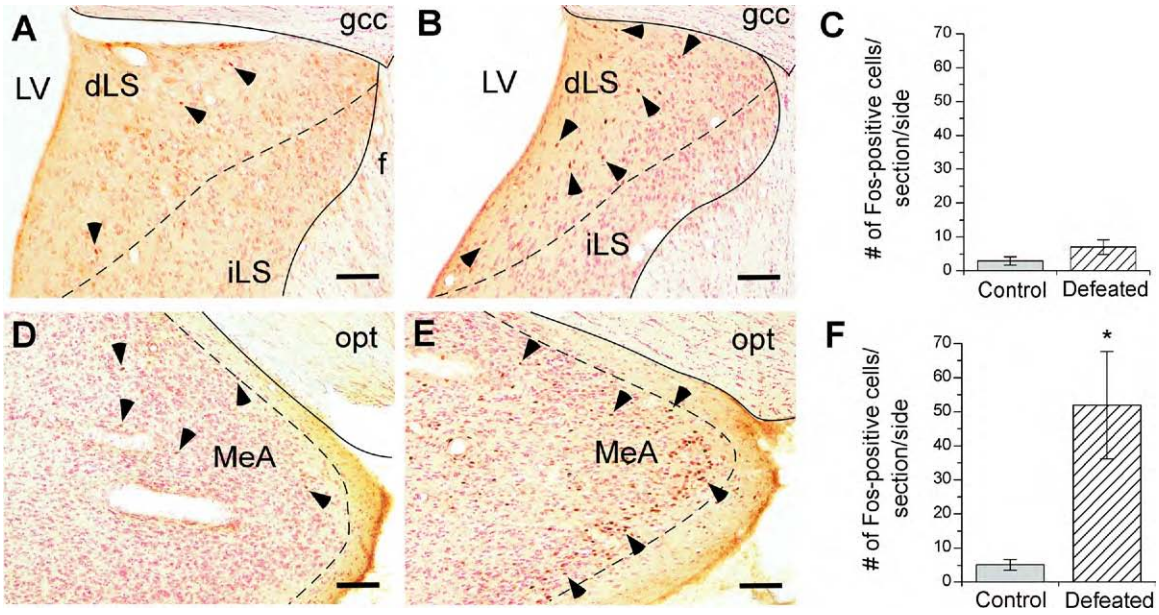

Fig. 1. Fos immunoreactivity in the dorsal lateral septum (A, B), and medial amygdala (D, E) 75 min after an acute defeat (B, $n=5$; E, $n=5$) or control (A, $n=6$; D, $n=6$) procedure. Dorsal LS and MeA panels are in the range of −0.26 to −0.30 and −2.80 to −3.14 mm from bregma, respectively. Bar graphs represent the mean (±SEM) number of Fos-positive cells per section per side in the dorsal lateral septum (C) and medial nucleus of the amygdala (F) after control (grey bars) or acute defeat (stripped bars) conditions. Selected Fos-positive cells are indicated with a black arrowhead. Symbols indicate significant difference between control and defeated rats: * $P<0.05$. Scale bar=100 μm. LV, lateral ventricle; gcc, genu of corpus callosum; iLS, intermediate part of the lateral septum; opt, optic tract.

In the hypothalamus, Fos-positive cells were consistently observed in the ARH and VMH (Table 1). The supraoptic nucleus and lateral hypothalamus showed few, but slightly increased numbers of Fos-positive cells after defeat. The PVN did not show Fos-positive cells 75 min post-defeat.

Quantification of Fos protein expression following acute social defeat

Fos-positive neurons were counted in selected brain regions 75 min after defeat. As shown in Figs. 1 and 2, compared with control rats, socially defeated rats showed

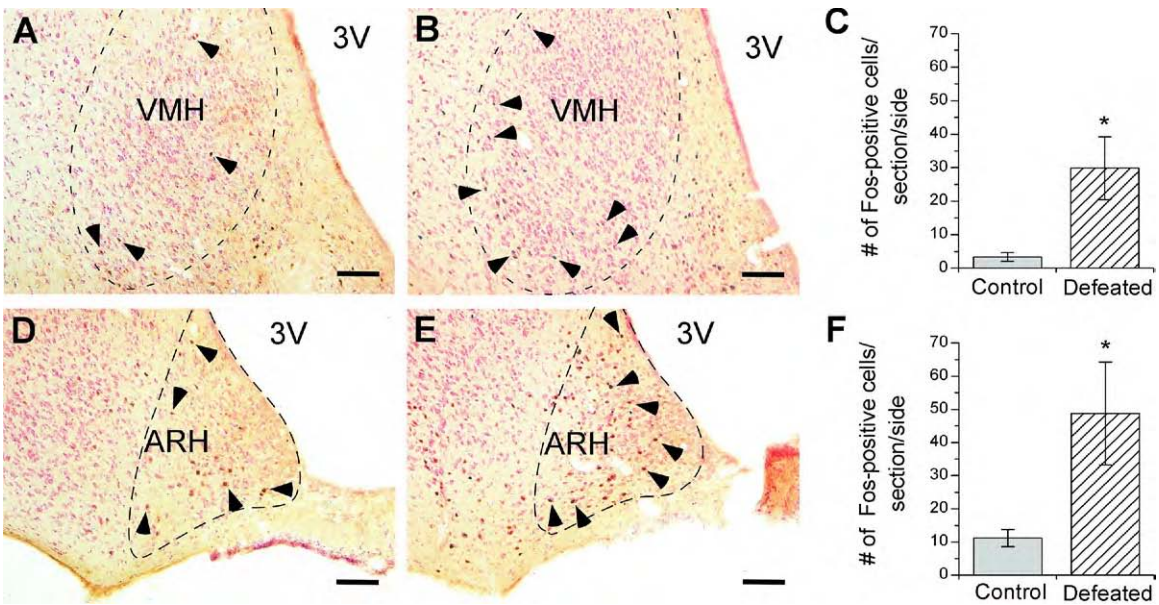

Fig. 2. Fos immunoreactivity in the ventromedial (A, B) and arcuate nuclei of the hypothalamus (D, E) 75 min after an acute defeat (B, $n=6$; E, $n=5$) or control (A, $n=6$; D, $n=5$) procedure. VMH and ARH panels are in the range of −2.30 to −3.14 and −2.30 to −2.56 mm (anterior/posterior) from bregma, respectively. Bar graphs represent the mean (±SEM) number of Fos-positive cells per section per side in the VMH (C) and the arcuate nucleus (F) after control (grey bars) or acute defeat (striped bars) conditions. Selected Fos-positive cells are indicated with a black arrowhead. Symbols indicate significant differences between control and defeated rats: * $P<0.05$. Scale bar=100 μm. 3V, Third ventricle.

significantly more Fos-positive cells in the MeA (Welch's t_4=2.97, $P \leq 0.05$) (Fig. 1D, E, F), especially the posterior part of the MeA. In the hypothalamus, defeat induced significantly more Fos-positive cells in the VMH (Welch's t_5=2.80, $P \leq 0.05$) (Fig. 2A, B, C) and ARH (Student's t_8=2.38, $P \leq 0.05$) (Fig. 2D, E, F). The majority of Fos immunostaining was evident in the ventrolateral part of the VMH and medial part of the ARH (Fig. 2B, E). No significant difference in Fos-positive cell counts was observed between control vs. defeated rats within the dLS (Fig. 1A, B, C), anterior BNST (defeated: 7.4±3.9, control: 7.2±2.8 cells), posterior BNST (defeated: 3.3±2.0, control: 9.5± 4.8 cells), or PVN (defeated: 8.0±2.9, control: 11.4±5.5 cells).

Partial colocalization of Fos protein and CRF_2 mRNA in the MeA

Similar to previous reports (Van Pett et al., 2000; Li et al., 2002), high levels of CRF_2 receptor mRNA were observed in the intermediate part of the LS (Supplementary Fig. 1A) and dorsomedial VMH (Supplementary Fig. 1C). Moderate CRF_2 mRNA density was observed in the dLS (Supplementary Fig. 1A), anterior and posterior MeA (Supplementary Fig. 1B), basomedial amygdala, and mainly in the posterior region of the BNST (not shown). Low levels of CRF_2 mRNA were also evident in the hippocampal formation (not shown), ARH (Supplementary Fig. 1D), magnocellular division of the PVN, and supraoptic nucleus (see Supplementary Table 1). The pattern of CRF_2 expression significantly overlapped with that of defeat-induced Fos expression, suggesting that social defeat might activate CRF_2-positive neurons in these areas. Therefore, dual-labeling of CRF_2 mRNA and Fos immunoreactivity was performed to determine whether some of the Fos-positive cells in these areas were also CRF_2-positive. As shown in Fig. 3D, E, and F, high degrees of colocalization of signals for Fos protein and CRF_2 mRNA were found in the MeA, in which 49.7% of Fos-positive cells expressed CRF_2 mRNA. Only moderate numbers (12.7%) of Fos-positive cells in the dLS were found to colocalize with CRF_2 mRNA (Fig. 3A, B, C). Negligible or no colocalization of CRF_2 mRNA with Fos protein was seen in the VMH (Fig. 3G, H, I; 14.2%) or ARH (Fig. 3J, K, L; 0.0%), respectively.

DISCUSSION

The present results indicate that CRF_2 receptor-expressing neurons in the MeA are involved in the response to acute defeat, a model of antagonistic social stress relevant to several affective and reproductive disorders (Luiten et al., 1985; Kollack-Walker and Newman, 1995; Martinez et al., 1998; Blanchard et al., 2001; Dominguez et al., 2001). Confirming previous findings that used c-*fos* mRNA or Fos protein expression as markers of neuronal activation, several brain areas were activated following social defeat (Cullinan et al., 1995). Fos protein expression 75 min post-defeat was evident in the MeA, LS, ARH, and VMH, structures which all express high levels of urocortin-CRF_2 system molecules (Van Pett et al., 2000; Li et al., 2002; reviewed in Fekete and Zorrilla, 2007). Combined *in situ* hybridization/immunocytochemistry demonstrated that a large proportion of Fos immunoreactive cells in the MeA, but not in the dLS, ARH, or VMH, were also positive for CRF_2 mRNA.

The MeA, which receives input from the vomeronasal and olfactory systems via the cortical nucleus of the amygdala (Newman, 1999), projects to brain regions involved in stress- and sociosexual reproduction-related functions, including other amygdala divisions, extended amygdala (e.g. BNST), hippocampus (Canteras et al., 1995), and hypothalamic nuclei that modulate neuroendocrine systems and sexual and agonist behaviors (e.g. medial preoptic area, VMH and ventral premammillary nucleus; Paredes and Baum, 1997). Accordingly, the MeA has a role in several social behavioral responses, including behavioral arousal (Kollack-Walker and Newman, 1995), acquisition and expression of conditioned defeat (Markham and Huhman, 2008), social learning and memory processes (Luiten et al., 1985), and fear and anxiety-like behavior (Chen et al., 2006). Relevant to defeat, MeA lesions prevent males from avoiding conspecifics that recently defeated them (Luiten et al., 1985) and severely impair mating, parental behavior (Sheehan et al., 2001), and other reproductive functions (Dominguez et al., 2001). Lesions of the MeA also reduce defensive responses to predator odor and escape responses to noxious stimuli (Blanchard et al., 2005). Additionally, the MeA is a proposed regulator of hypothalamic–pituitary–adrenal axis activity (Dayas et al., 1999), with MeA stimulation increasing plasma glucocorticoid and adrenocorticotropic hormone levels (Herman et al., 1996). The results raise the hypothesis that CRF_2-synthesizing neurons in the MeA may participate in consequences of, or counter-regulatory responses to, defeat.

As a structure subserving sociosexual and agonistic behavior, the MeA is structurally and functionally sexually dimorphic (Cooke and Woolley, 2005; Cooke, 2006), with the posterodorsal MeA ~50% larger in males than in females (Hines et al., 1992) in relation to circulating androgen levels. The MeA has many neurons that contain high levels of estrogen and/or testosterone and their receptors (Greco et al., 1998), a finding specifically true of CRF_2-expressing neurons in the posterior MeA (Van Pett et al., 2000). Stressors, including defeat, suppress testosterone secretion in both rodents and primates, including man (Armario and Castellanos, 1984; Sapolsky, 1985; Schultheiss et al., 2005). Perhaps the defeat-induced changes in CRF_2-expressing MeA circuitry are associated with the suppression of circulating testosterone.

A candidate natural ligand for MeA CRF_2 receptors is urocortin 3. Many urocortin 3–positive neurons and fibers are present in the MeA, where its expression is increased by stress (Jamieson et al., 2006), and in the cortical nucleus of the amygdala, from which the MeA receives extensive projections (Li et al., 2002). Several studies suggest an anxiolytic-like role for CRF_2 receptors. For example, administration of urocortin into the adjacent CeA reduced anxiety-like behavior during ethanol withdrawal (Valdez et al., 2004). I.c.v. urocortin 3 administration also

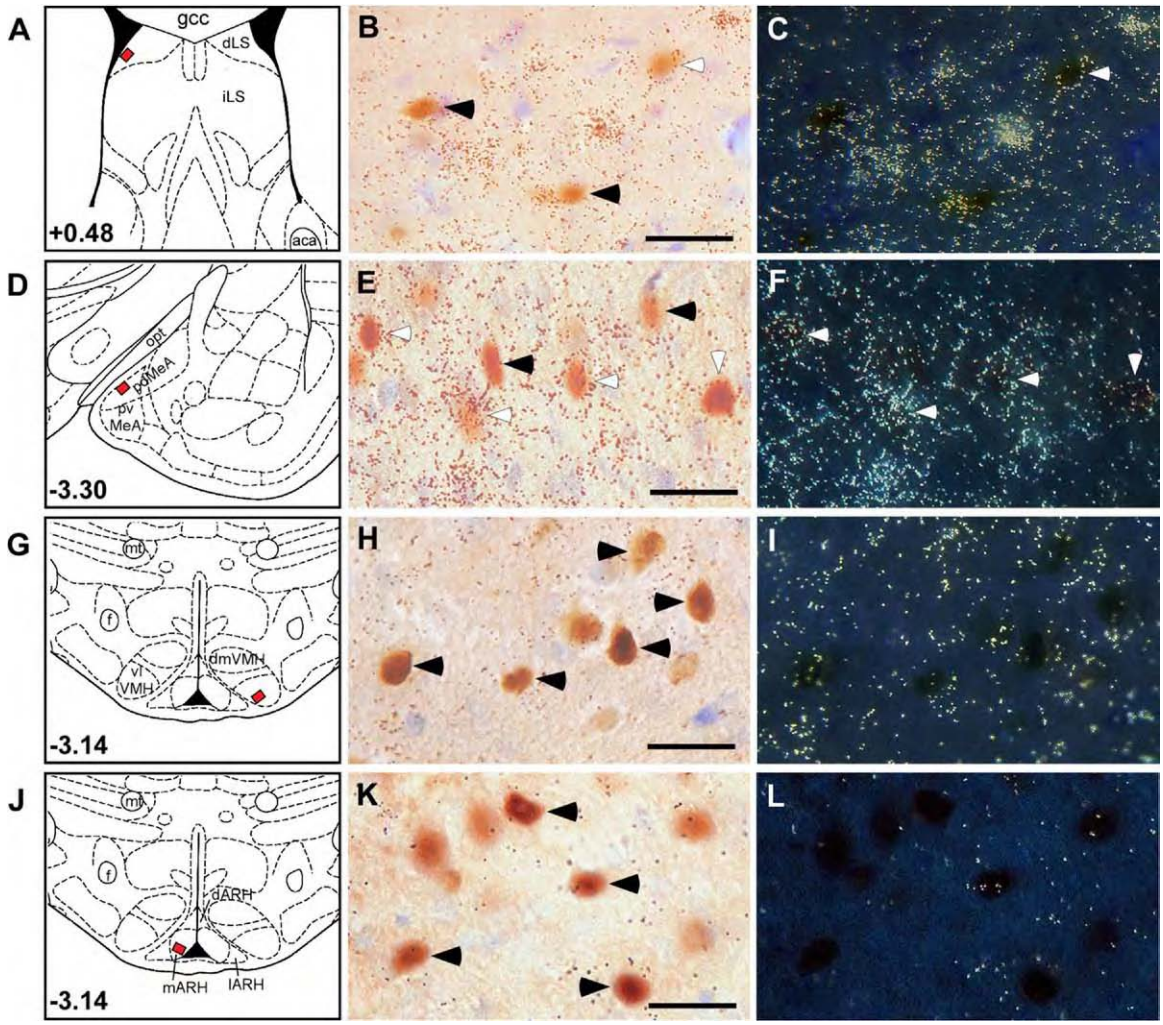

Fig. 3. Acute defeat induces Fos protein expression at sites of CRF_2 receptor mRNA expression in the rat brain. Schematic panels showing the bregma level (A, D, G, J) with the small black square filled with red within them indicating the region photographed at higher magnification and shown in B, C, E, F, H, I, K, and L. Brightfield photomicrographs of sections through the dorsal lateral septal (B), posterior medial amygdala (E), ventrolateral ventromedial hypothalamus (H), and medial arcuate nuclei (K) of the hypothalamus showing cells with acute defeat-induced nuclear Fos immunoreactivity and highlighted with silver grain clusters (dark dots), indicating a positive CRF_2 receptor mRNA signal (white arrowheads). Darkfield photomicrographs of adjacent sections (C, F, I, L) compare the distribution of CRF_2 mRNA (bright small dots, as clusters of silver grains, indicating [^{33}P]uridine triphosphate-labeled CRF_2 cRNA probe signals). Cells labeled singly for Fos (black arrowheads) or the CRF_2 transcript colocalizing with Fos-labeled cells (white arrowhead) are shown for contrast. gcc, Genu of corpus callosum; iLS, intermediate lateral septum; aca, anterior part of anterior commissure; opt, optic tract; pd or pvMeA, posterodorsal or posteromedial medial amygdala; mt, mammillothalamic tract; f, fornix; vl or dmVMH, ventrolateral or dorsomedial ventromedial nuclei of hypothalamus; d or m or lARH, dorsal or medial or lateral arcuate nuclei of the hypothalamus. Scale bar=50 μm.

reduced anxiety-like behavior in some, but not all, rodent models of anxiety-like behavior (Zhao et al., 2007). Some, but not all (Coste et al., 2000), studies of CRF_2 knockout mice observed an anxiogenic-like phenotype of mice following stressor exposure (e.g. in the elevated plus maze [Bale et al., 2000; Kishimoto et al., 2000], emergence, open field [Bale et al., 2000], or light/dark box tests [Henry et al., 2006]). Conversely, some findings (Pelleymounter et al., 2004) support an alternate hypothesis that CRF_2 receptor activation has anxiogenic-like effects, with lateral septal (Henry et al., 2006) or dorsal raphe (Hammack et al., 2003) CRF_2 activation promoting defensive responses to uncontrollable stressors. However, to our knowledge, no studies have evaluated the behavioral effects of intra-MeA CRF_2 agonist infusion. Thus, the specific anxiety-related or other behavioral role of CRF_2 receptors in the MeA remains to be determined.

Defeat also increased the number of Fos-positive cells in the VMH, another androgen receptor–expressing nucleus that is larger in males than in females (Dugger et al., 2007). However, induction of Fos immunoreactivity was observed mostly in the ventrolateral VMH, whereas CRF_2 mRNA predominated in the dorsomedial VMH, similar to previous studies (Lovenberg et al., 1995). Functional segregation of the dorsomedial vs. ventrolateral VMH has been proposed previously. For example, in males, the dorsomedial VMH is critical for ultrasonic vocalization and scent marking behaviors (Harding and McGinnis, 2005),

whereas the ventrolateral VMH subserves mounting behavior (Pfaff and Sakuma, 1979). The dorsomedial VMH also putatively differentially subserves energy homeostasis (Flanagan-Cato, 2003), defensive aggression (Canteras et al., 1994; Canteras, 2002), and innate affective reactions to pain (Borszcz, 2006). The two VMH subregions receive innervation from topographically distinct aspects of the MeA (Canteras et al., 1995). The ventrolateral VMH also more closely innervates other hypothalamic regions that express gonadal steroid hormone receptors, including the medial preoptic, tuberal, and ventral premammillary nuclei, whereas the dorsomedial VMH differentially projects to anterior hypothalamic and dorsal premammillary nuclei (Canteras et al., 1994). Finally, the androgen-dependent sexual dimorphism of VMH volume is seen selectively in the ventrolateral, but not dorsomedial, subdivision (Dugger et al., 2007). The results suggest that defeat-induced Fos protein expression in the VMH may involve a subpopulation of cells distinct from those that express CRF_2 receptors.

Defeat also induced Fos protein expression in a subpopulation of cells in the medial ARH distinct from those that synthesize CRF_2 receptors. Neurons in the medial ARH have a role in regulatory inhibition of the hypothalamic–pituitary–adrenal axis (Kollack-Walker et al., 1997) and of prolactin secretion via tuberoinfundibular dopamine projections to the median eminence (Freeman et al., 2000). Lesions of the ARH increased basal glucocorticoid levels and enhanced adrenocortical activity in response to stress (Cullinan et al., 1995). A separate population of mediobasal ARH neurons has recognized roles in energy homeostasis and reproductive functions via parallel ascending projections of orexigenic neuropeptide Y/agouti-related protein-expressing neurons and anorexigenic proopiomelanocortin/cocaine and amphetamine-regulated transcript-expressing neurons to other hypothalamic nuclei (Hill et al., 2008). Arcuate neurons also are implicated in opioid-mediated stress-induced analgesia (Wang et al., 1990) following defeat (Miczek et al., 1985). Identification of the phenotypes of Fos-positive cells in the ARH may provide further insight into the physiological role and function of ARH neurons in social defeat stress.

The absence of PVN or BNST Fos protein expression following defeat stress was somewhat unexpected (Herman et al., 1996; Nail-Boucherie et al., 1998). The time course of analysis may be relevant, with PVN and BNST Fos expression perhaps occurring transiently and earlier in time. Indeed, in separate studies, increased Fos protein expression was observed in both the PVN and BNST 30 min post-defeat (ÉM Fekete, Y Zhao, C Li, V Sabino, WW Vale, EP Zorrilla, unpublished observations). Nonetheless, the present findings are consistent with reports that hypothalamic–pituitary–adrenal axis activation did not require PVN Fos expression (Brown and Sawchenko, 1997; Figueiredo et al., 2003) and that c-*fos* mRNA was not elevated in the LS, PVN, or BNST following defeat, while it was seen after foot shock or restraint (Funk et al., 2006). The functional significance of early BNST activation after defeat is similarly unclear. On the one hand, the BNST has been associated with fear-related behavior (Lee and Davis, 1997; Onaka and Yagi, 1998), and injection of antisauvagine-30 (a preferential CRF_2 antagonist) into the BNST reduced conditioned defeat behavior (Cooper and Huhman, 2005). On the other hand, the number of threats received by an intruder correlated negatively with *c-Fos* expression in the BNST in a previous study (Martinez et al., 1998).

Importantly, c-*fos* activation does not provide a complete map of all neurons activated during a given stimulus (Robertson et al., 1989). Some activated neurons that express Fos protein may lead to downstream inhibition, and Fos protein is not known as a good marker of inhibition. Thus, induction of Fos immunoreactivity provides positive identification of brain areas activated during social conflict, but the absence of such labeling does not necessarily equal lack of involvement. Studies with other functional markers may help further delineate brain activation and inhibition patterns associated with social defeat. The present findings show, however, that CRF_2-synthesizing neurons in the posterior MeA are part of the neural response to social defeat.

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APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.neuroscience.2009.03.078.

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Neuroadaptations in Stress-Related Peptidergic Brain Systems Following Social Defeat Stress

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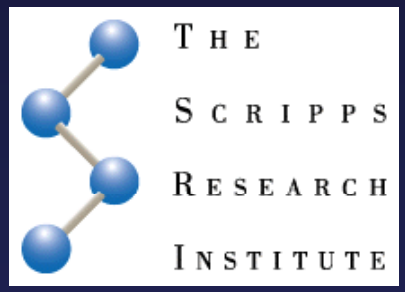

Zorrilla EP, Fekete EM, Zhao Y, Logrip M, Cottone P, Li C, Vale W, Sabino V

Disclosures

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Off-Label Usage: None
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Objectives

To further characterize an animal model of PTSD symptoms

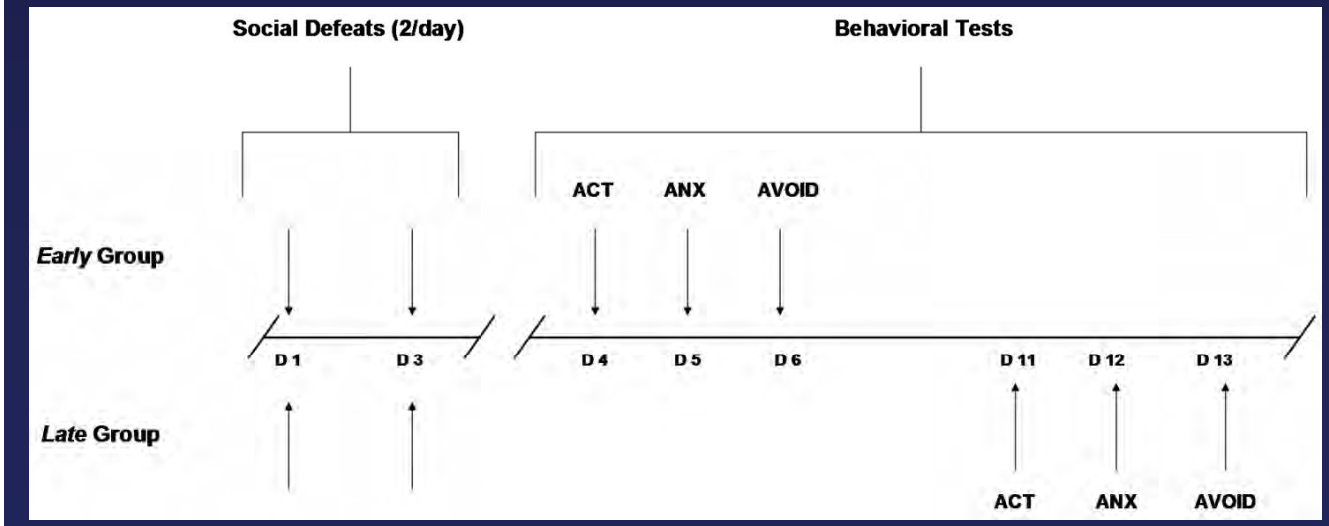
To identify associated gene expression changes in extrahypothalamic stress and reward neuropeptide systems

- amygdala, n. accumbens
-corticotropin-releasing factor (CRF), dynorphin-kappa opioid receptor (KOR),

To study therapeutic potential of nonpeptide CRF antagonists in model (ongoing)

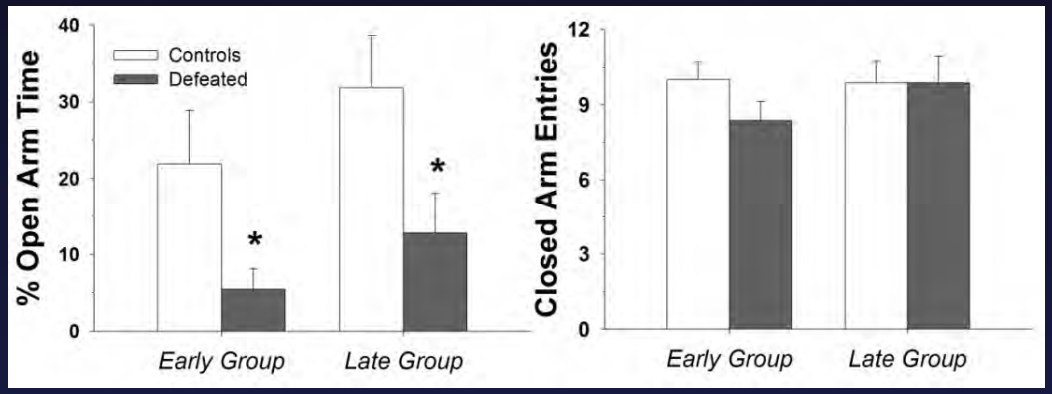
Repeated Social Defeat Model

"Resident-intruder" procedure
Unfamiliar territory
Threat of physical injury

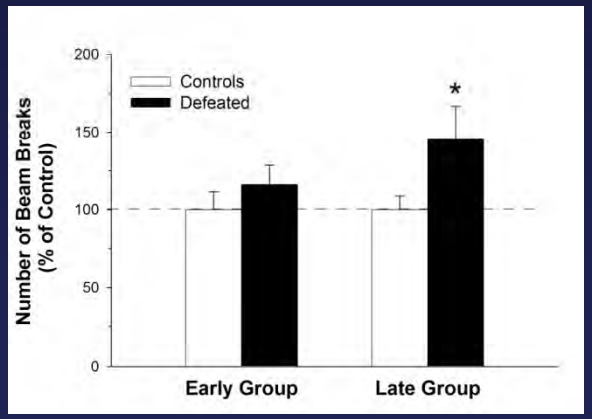


Selected Effects of Repeated Social Defeat

Persistent anxiety-like behavior (plus-maze)



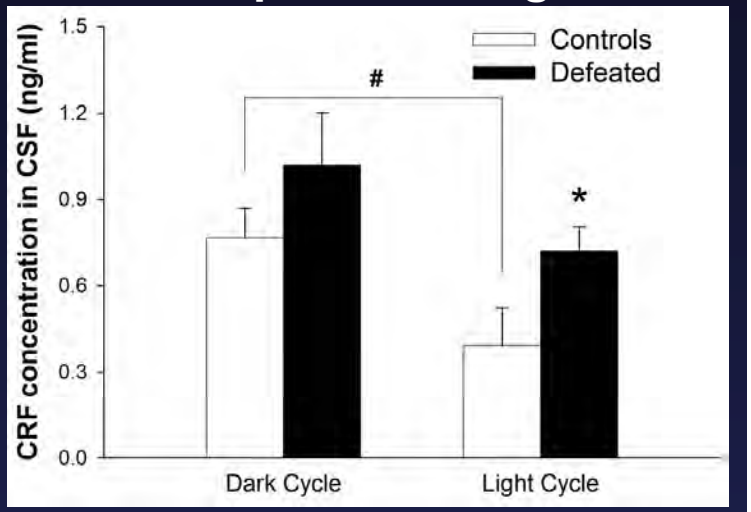
Emerging sleep-phase activity



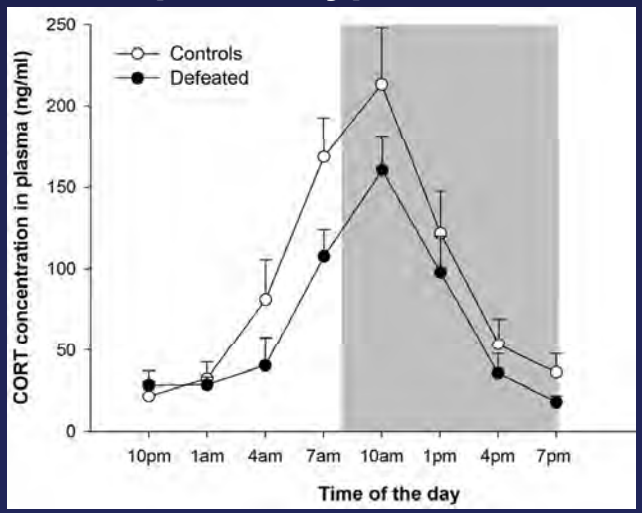
- Avoidance of defeat-associated cue (odor)

- Hypohedonia: Chronic imipramine-reversible reductions in willingness to work to obtain sweet solution

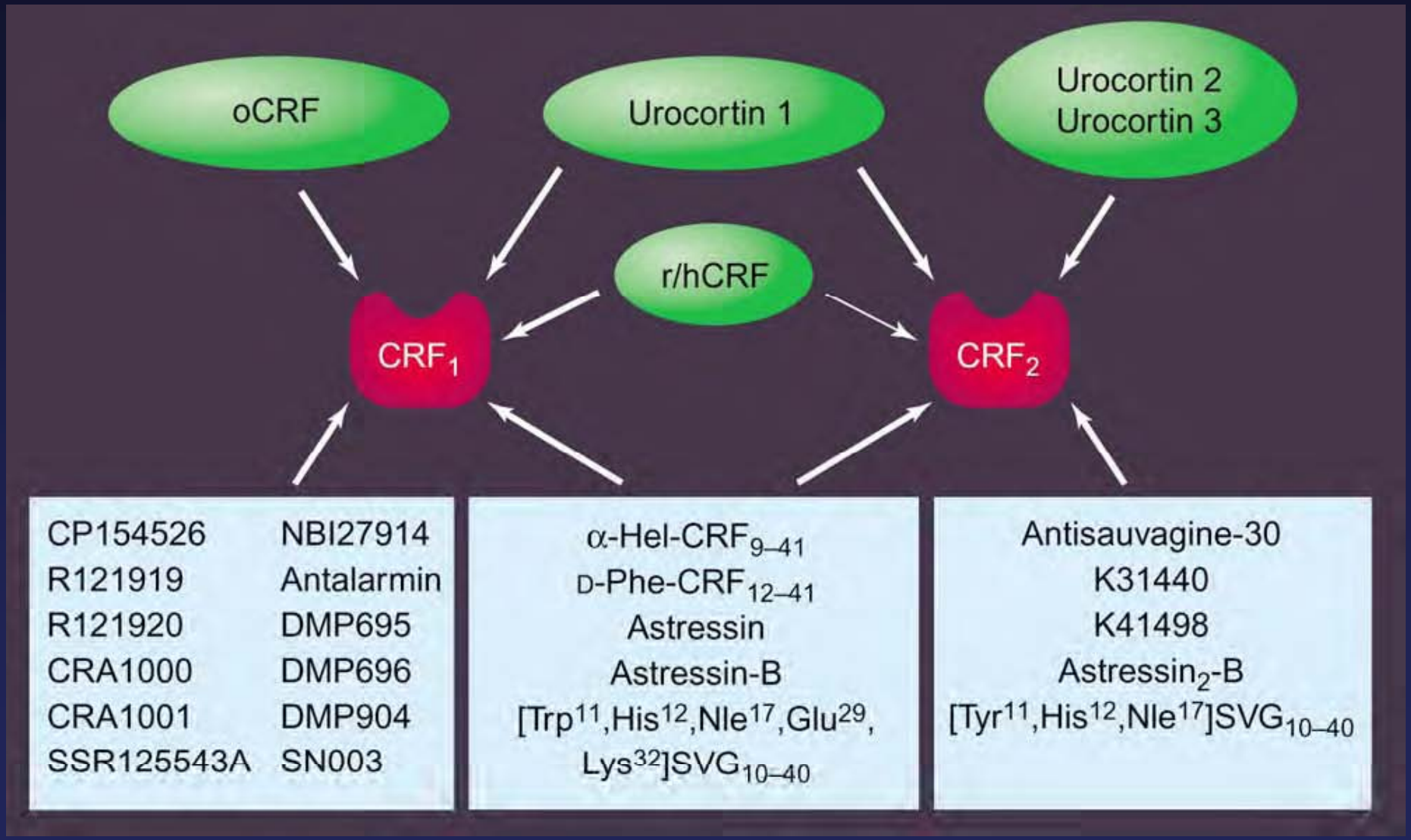
Increased CSF corticotropin-releasing factor



Blunted circadian CORT amplitude: Active phase hypocorticism



Mammalian CRF Receptors, their Putative Natural Ligands and Synthetic Receptor Antagonists

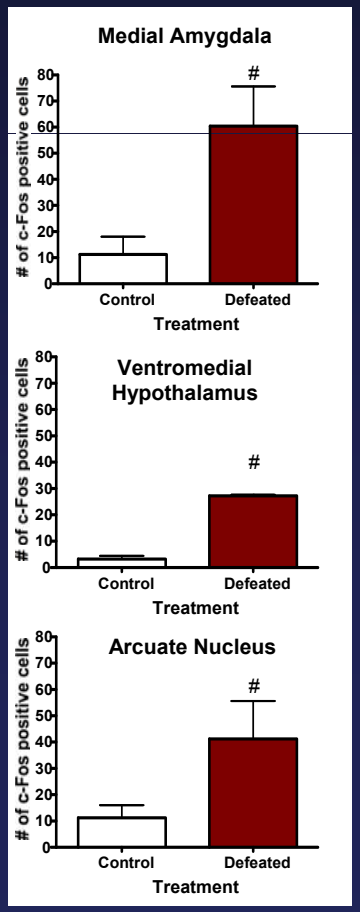


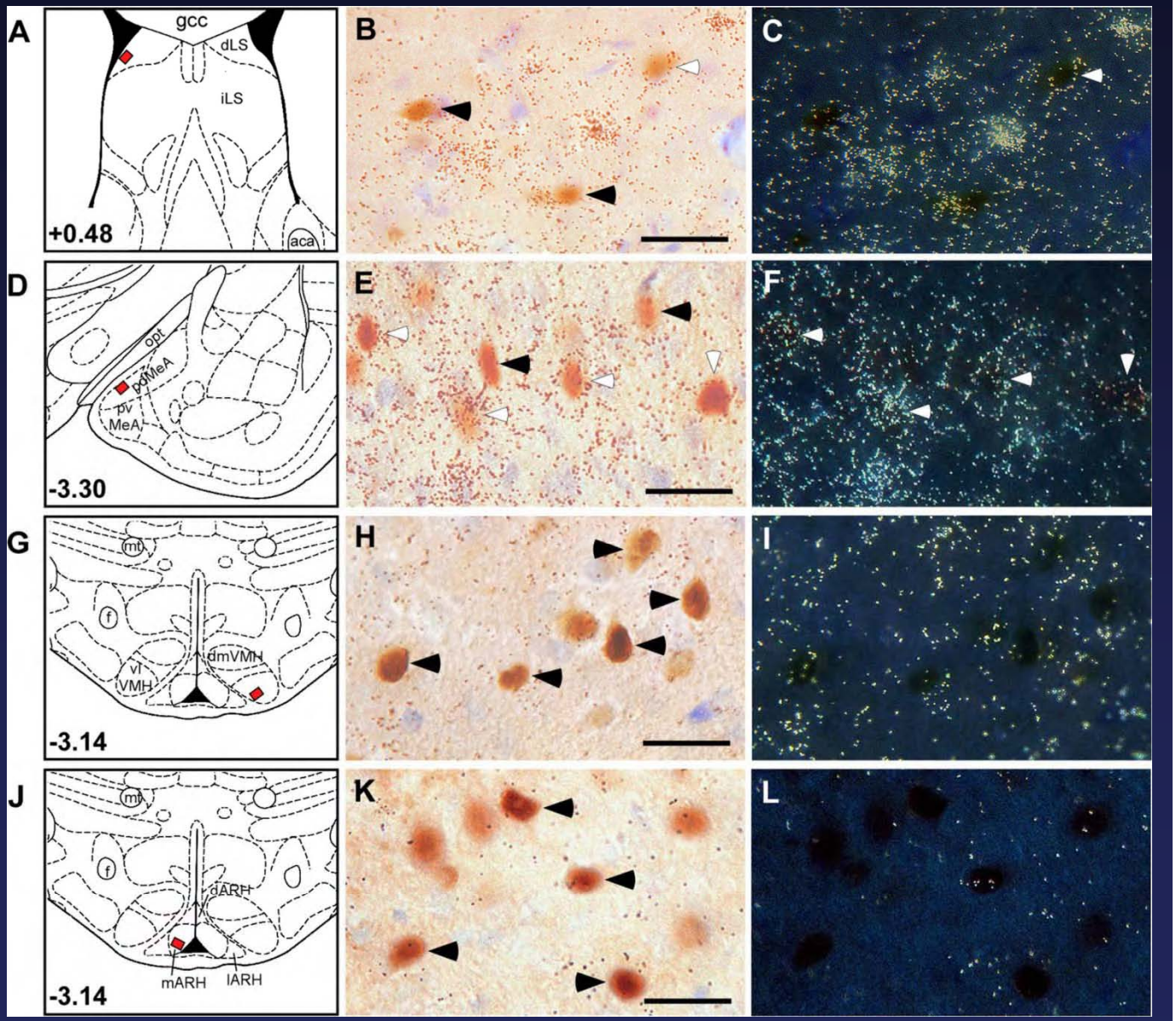
From: Zorrilla EP, Taché Y and Koob GF, 2003, *Trends Pharmacol Sci*, 24:421-427.

Defeat-induced c-Fos in CRF2-expressing regions: Co-localization in medial amygdala

Lateral Septum

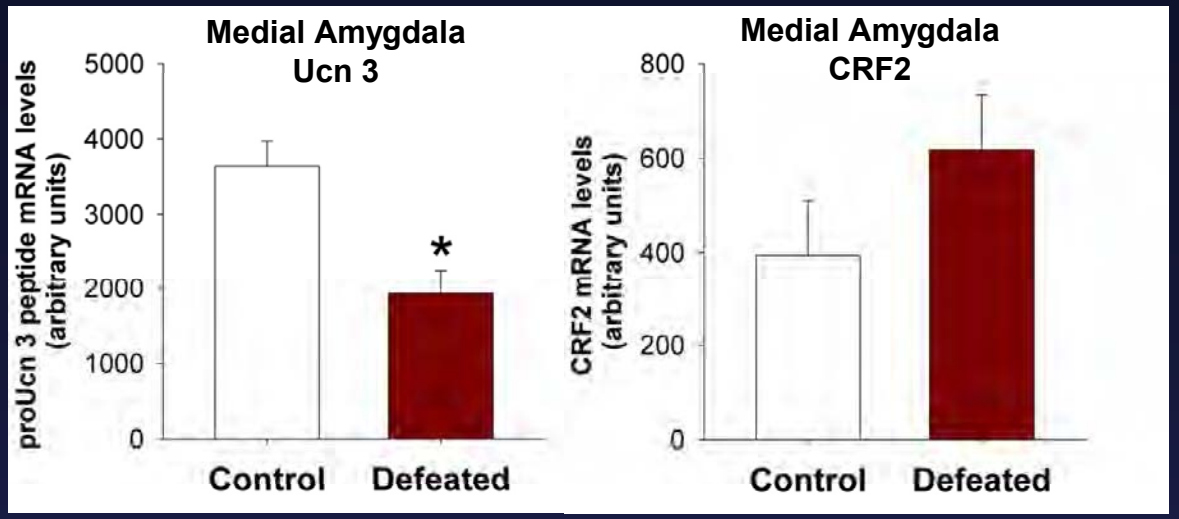
Not significant

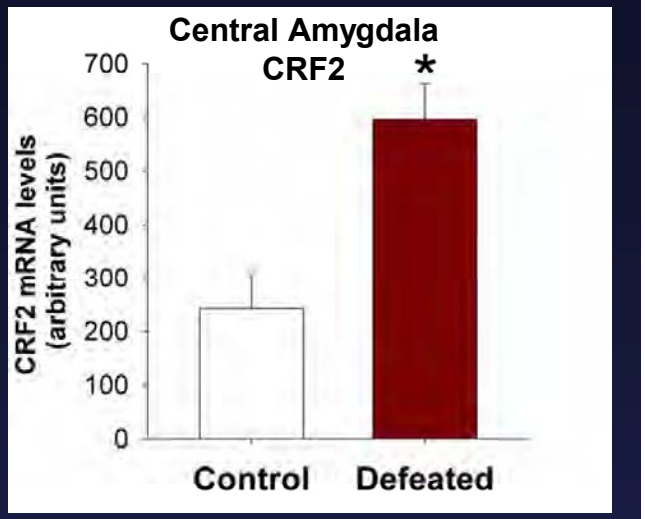




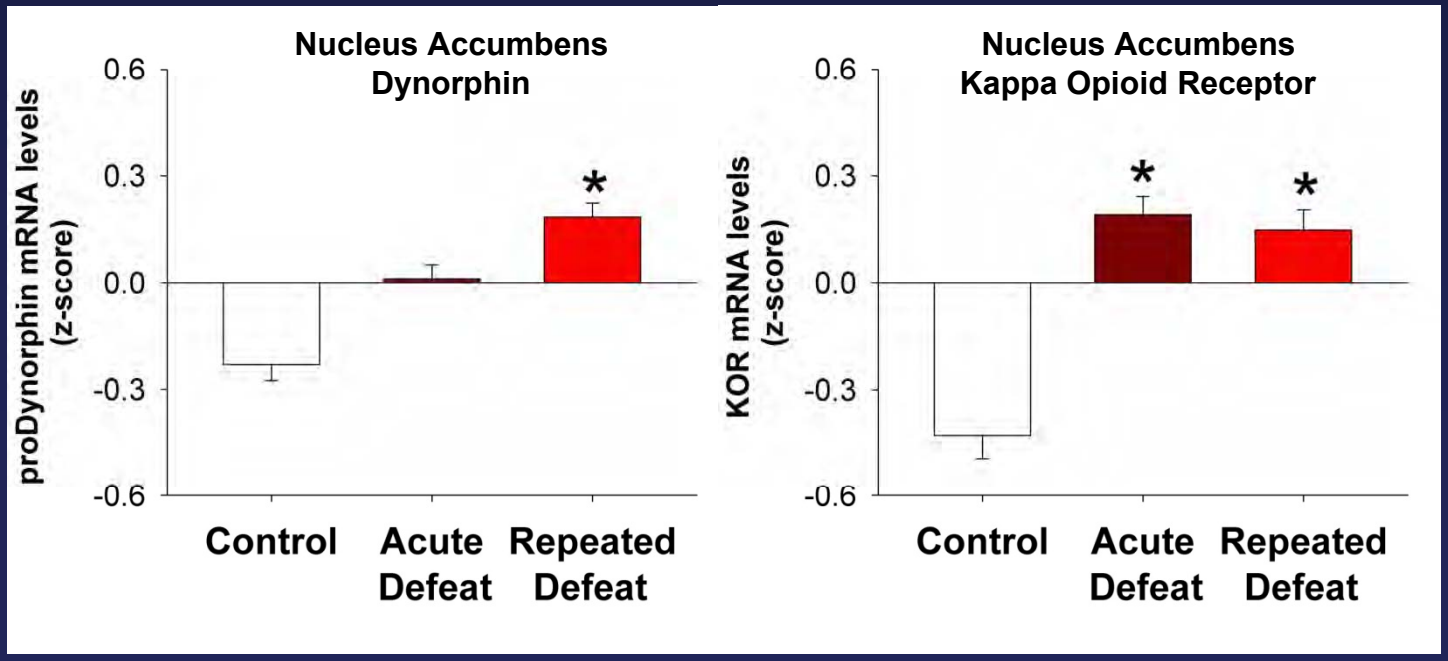
From: Fekete et al., 2009, *Neuroscience*, 162, 5-13.

Defeat alters amygdala Ucn 3–CRF2 system gene expression





Defeat increases n. accumbens KOR system gene expression



Summary and Impact

Repeated social defeat in rats elicits persistent behavioral (anxiety, anhedonia, avoidance, activation during sleep) and neuroendocrine (CRF, CORT) signs that resemble effects of post-traumatic stress.

The studies implicate 1) increased central CRF activity, 2) altered amygdala Ucn 3-CRF_2 signaling, and 3) increased accumbens dynorphin-KOR activity in effects of defeat.

The functional significance of gene expression differences is being explored using site-specific administration of subtype-specific ligands.

Ongoing studies may ultimately show that CRF or KOR system ligands have therapeutic potential for PTSD.

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